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Abstract: This study focuses on characterization of the cardiac N-methyl D-aspartate receptors (NMDARs) as a target for endogenous and synthetic agonists and antagonists. Using isolated perfused rat hearts, we have shown that intracoronary administration of the NMDAR agonists and antagonists has a pronounced effect on autonomous heart function. Perfusion of rat hearts with autologous blood supplemented with NMDAR agonists was associated with induction of tachycardia, sinus arrhythmia and ischemia occurring within physiological plasma concentration range for glutamate and glycine. Intracoronary administration of the NMDAR antagonists exerted an anti-arrhythmic effect and resulted in bradycardia and improvement of capillary perfusion. Action of antagonists eliprodil, Ro25-6981, memantine, ketamine, and MK-801 on autonomous heart function diverged strikingly from that of L-type Ca channel blockers. Cardiac NMDAR subunit composition differed from that of neuronal receptors and was age- and chamber-specific. Transcripts of the GluN3A and GluN2D were found in all heart chambers, whereas expression of GluN1 and GluN2A and 2C were restricted to the atria. Expression of the GluN2B protein in ventricles increased markedly with age of the animals. The obtained data reveal that NMDARs are expressed in rat heart contributing to the autonomic heart rate regulation and the function of the cardiac conduction system.

DOI: https://doi.org/10.1097/FJC.0000000000000424

Journal of Cardiovascular Pharmacology Publish Ahead of Print
DOI: 10.1097/FJC.0000000000000424

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Short running title: Cardiac N-Methyl D-aspartate receptors

Funding and acknowledgements

This study was supported by the SNF grants 1112 449 and 310030_124970/1 to AB and by Saarland University's internal funding program HOMFOR (to QT and LK).

We thank Alexander Kosenkov for his help with analysis of autoradiogaphic images.

Abstract

This study focuses on characterization of the cardiac N-methyl D-aspartate receptors (NMDARs) as a target for endogenous and synthetic agonists and antagonists. Using isolated perfused rat hearts, we have shown that intracoronary administration of the NMDAR agonists and antagonists has a pronounced effect on autonomous heart function. Perfusion of rat hearts with autologous blood supplemented with NMDAR agonists was associated with induction of tachycardia, sinus arrhythmia and ischemia occurring within physiological plasma concentration range for glutamate and glycine. Intracoronary administration of the NMDAR antagonists exerted an anti-arrhythmic effect and resulted in bradycardia and improvement of capillary perfusion. Action of antagonists eliprodil, Ro25-6981, memantine, ketamine, and MK-801 on autonomous heart function diverged strikingly from that of L-type Ca^{2+} channel blockers. Cardiac NMDAR subunit composition differed from that of neuronal receptors and was age- and chamber-specific. Transcripts of the GluN3A and GluN2D were found in all heart chambers, whereas expression of GluN1 and GluN2A and 2C were restricted to the atria. Expression of the GluN2B protein in ventricles increased markedly with age of the animals. The obtained data reveal that NMDARs are expressed in rat heart contributing to the autonomic heart rate regulation and the function of the cardiac conduction system.

Key words: N-methyl D-aspartate receptors, heart, heart rate, arrhythmia, antagonists

Introduction

The role of N-methyl D-aspartate receptors (NMDARs) in brain development, cognitive function and progression of neurological disorders cannot be overestimated. These ligand-gated cation channels formed by glycine-binding (GluN1 and GluN3A/B) and glutamate-binding (GluN2A, B, C and D) subunits show high preference for conducting Ca^{2+} (1). Gating properties, conductance, deactivation kinetics as well as responses to inhibitors and allosteric modulators vary greatly depending on the subunit composition of the receptor complex (2). The pivotal importance of this class of ionotropic glutamate receptors in the brain gave rise to a rapid progress in pharmacology during the past 30 years and resulted in development of numerous antagonists targeting specific subunits that form the receptor or specific binding sites (3).

These compounds are currently used for medication of patients with Alzheimer's disease, Huntington's disease, and various forms of dementia. Some of them are broadly used as anaesthetics and others were tested for treatment of epilepsy and stroke (4). Each antagonist used to target the NMDARs in the central nervous system has been shown to produce multiple systemic effects. In particular, compounds targeting 1-(1-phenylcyclohexyl) piperidine (phencyclidine, PCP)-binding site within the receptor channel pore (e.g. memantine, ketamine, and MK-801) were reported to act as antiarrhythmic drugs in animal models (5-7). The PCP-targeting antagonist aptiganel HCl decreased the incidence of arrhythmia, suppressed premature ventricular complexes (PVCs) and reduced the S-T interval depression in human patients affected by stroke (4). Eliprodil, another NMDAR antagonist interacting selectively with the glutamate-binding GluN2B subunit, was shown to modulate oxygen consumption in the heart (8, 9) and cause prolongation of the rate corrected QT interval (QTc) in human subjects at high doses (4). Initially, all the changes in the heart function triggered by the NMDAR agonists and antagonists were attributed to the action of these compounds on the central and peripheral nervous system. Indeed, microinjections of NMDA or glutamate directly into nucleus tractus solitaries caused bradycardia and reduced arterial pressure in rats. These

effects were inhibited by the receptor antagonists MK-801 and AP5 (10, 11). The GluN1, GluN2A, and GluN2B subunits were detected in vagal preganglionic neurons projecting to the rat heart (12). However, the effects of agonists and antagonists of the NMDARs were retained in denervated hearts. MK-801 in rat and ketamine in guinea pig spontaneously beating right atria caused a dose-dependent reduction in heart rate (HR) and contractile force (13) (14). Similar negative inotropic and chronotropic effects were observed in isolated hearts from rabbits and guinea pigs perfused with Krebs-Ringer or Tyrode buffer supplemented with ketamine (15, 16). Identification of the localization and molecular identity of targets for action of these compounds may prove useful. Homocysteine is acknowledged as an agonists of the NMDARs and a cardiovascular risk factor (17). Administration of NMDA intraperitoneally for two weeks resulted in development of ventricular arrhythmias in rats that could be prevented by co-administration of MK-801 (18). These findings suggest that the NMDARs may play a role in development of arrhythmias and hence may be considered a potential pharmacological target. Whether these receptors are localized in the central nervous system or in the heart remains to be clarified.

Recently, expression of the GluN1 and GluN2B subunits of the NMDARs was confirmed in heart tissue of several mammalian species (19-21). However, detailed characterization of autonomous heart responses to the NMDAR agonists and antagonists was lacking. Subunit composition of cardiac NMDAR complexes, pharmacology of cardiac NMDARs, and kinetics of responses of the heart to the alterations in receptor activity remained unidentified. This study presents detailed characterization of autonomous responses of the isolated blood-perfused rat heart to agonists and antagonists of the NMDARs. Using autoradiography and qPCR the heart was mapped for the NMDAR activity and subunit composition and the specific interaction of antagonists with cardiac NMDARs was explored. The obtained results indicate that cardiac NMDARs actively participate in regulation of HR and heart rate variability (HRV).

Materials and methods

2.1 Animals, anesthesia and organ harvesting

All experiments were conducted in accordance with Swiss animal protection laws and institutional guidelines that comply with guidelines of the American Physiological Society and the Institute of Laboratory Animal Resources and approved by the Veterinary Office of Canton Zurich (animal experimentation permit number #60/2011). Wistar rats of both sex, aged from 0.5 months to 2 years

were used for the study. Animals were purchased from Janvier Labs (Le Genest St Isle, France). For the organ harvesting rats were anesthetized using 3% isofluorane applied in the even O_2/N_2O mixture (800 ml/min). The abdominal cavity was opened and heparin (Heparin-sodium, Braum, 5000U/kg) administered into caudal vena cava. Subsequently 8-10 ml of blood were withdrawn and hearts were harvested and placed into the ice-cold perfusion buffer containing (in mM): 120 NaCl, 25 $NaHCO_3$, 5 KCl, 10 glucose, 10 TRIS-HCl, 1 $CaCl_2$, 0.15 $MgCl_2$, pH 7.4. In 2-3 min time hears were mounted on a cannule and perfused though the aorta with autologous blood, human RBC suspension, or with Krebs-Henseleit (KH) buffer containing (in mM) 118.5 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 1.25 $CaCl_2$, 1.2 KH_2PO_4, 25 $NaHCO_3$, 0.1 L-arginine, 2 pyruvate, 0.1 taurine and 11 glucose, pH 7.4 at 37°C. As a result of short-term global ischemia followed by reperfusion during the heart harvesting 33 out of 96 young hearts (rats aged 3-6 month) immediately developed sinus arrhythmia (see Table 1) and 5 out of 36 old hearts (rats aged 1-2 years) were presented with ventricular fibrillation that started during the restitution period.

2.2 Ex vivo blood-perfused rat heart model

The organ perfusion circuit used for the rat heart perfusions is consisted of mini-oxygenator, thermostated organ chamber and peristaltic pump with total volume of recirculating liquid of 10 ml, 3 ml/min perfusion speed (22). The small volume of the circuit allows using autologous rat blood. Blood-perfused rodent hearts showed better myoglobin saturation by oxygen and thus allow to preserve cardiac function, while in the buffer-perfused heart regional hypoxia was observed ((23), (24), (25)). Thus, the blood-perfused circuit was the main model used at the present study. The perfusion protocol was described somewhere in details (22). In brief, the perfusion circuit was pre-filled with perfusion media containing (in mM): 120 NaCl, 25 $NaHCO_3$, 5 KCl, 10 glucose, 10 TRIS-HCl, 1 $CaCl_2$, 0.15 $MgCl_2$, pH 7.4, then the whole rat blood was added to the final hematocrit value inside the circuit of 20-25%. Mini-oxygenator fibers were subjected to humid mixture of 95% O_2 / 5% CO_2, resulting in blood oxygen saturation of 98% (normoxic condition). 5%O_2, 5%CO_2 and 90% N_2 gas mixture was used to simulate hypoxia (SO_2 35%, (22)). Drugs and treatments were applied after 15-20 min restitution period after the stabilization of hear contractions. All perfusions were terminated after 1h and heart tissue was harvested.

To reduce amount of plasma-borne NMDAR agonists, washed human red blood cells (RBCs) were used in a separate set of experiments in which dose-response of the changes in heart rate (HR), heart rate variability (HRV), and ECG were measured. Diameter of human RBCs is similar to that of rat

RBCs, thus they are a good substitution of the rat blood for rat heart perfusions (unlike bovine or goat RBCs that are smaller in size, (26)). Moreover, human red blood cells are more resistant to the mechanical stress applied by the peristaltic pump compare to rodent red blood cells. Thus, in this setting we also reduced a release of erythrocyte-born amino acids into the perfusion fluid (plasma glutamate concentration is around 20-50 µM, while intraerythrocytic concentration is about 0.5 mM (27). Human blood was collected from the vein of healthy donors into heparinized vacutainers. Blood sampling was performed at the University Hospital Zurich on written consent. Blood collection for was approved by the cantonal ethic committee and conforms to the principles outlined in the Helsinki Declaration. RBCs were washed 6 times and were finally resuspended in the buffer containing (in mM): 120 NaCl, 5 KCl, 25 $NaHCO_3$, 2 $CaCl_2$, 0.15 $MgCl_2$, 10 TRIS-HCl, 10 glucose, 2 pyruvate, 0.1 arginine, 0.1 taurine, pH 7.4 to 25% hematocrit and filled into the heart perfusion circuit. Reoxygenation of the RBC suspension in the buffer was performed using humidified 95% O_2 and 5% CO_2 gas mixture at 37 ^{0}C.

To avoid further contaminations from the circulating red blood cells and accumulation of the heart-borne compounds released into the circuit, hearts were perfused with KH buffer equilibrated with

gas phase containing 95% O_2 and 5% CO_2 and warmed to 37^{0}C in a non-recirculating manner in the

special set of experiments. After the 15 min restitution period hearts were supplemented with 1:1 glutamate/glycine mixture (0-500 µM each) with or without 50 µM MK-801. Heart rate, heart rate variability and ECG were monitored and analyzed as described below.

Intracoronary administration of a selection of agonists and antagonists was used to explore the biochemistry and pharmacology of cardiac NMDARs. Selection was performed to overcome the lack of specificity of each individual blocker or agonist (with exception for N-methyl D-aspartate (NMDA), and D-Serine). Although most of the compounds tested had multiple targets, NMDAR was the only target shared by all of them (Table 2 and supplementary Table 1, http://links.lww.com/JCVP/A240). Thus, we used stock solutions of the agonists (NMDA, glutamate, glycine, homocysteic acid (HCA), D-serine), and antagonists (PCP binding site): memantine, MK-801, ketamine; polyamine binding site-targeting antagonists: eliprodil, Ro25-6981; and $ZnCl_2$), glutamate binding site (D-APV) and glycine binding site (L-701). The concentrations of

agonists and antagonists were chosen based on their affinities to the neuronal NMDARs reported elsewhere (28, 29). L-type calcium channel blockers (CCBs) diltiazem, and verapamil were always used at concentrations, 10 μM and 5 μM respectively.

2.3 ECG recordings, HR, HRV

During the perfusion ECG electrodes were fixed vertically on either side of the hearts so that the resulting recordings corresponded to the aVL projection. Due to high inter-individual variability, all observed effects were compared with baseline values from the same heart.

Heart rate was calculated from the RR intervals extracted from the ECG or as a peak-to-peak interval. Analysis was performed using the MLS360/7 ECG Analysis Module of the LabChart 7 Pro Software (ADInstruments Ltd). The values averaged over the last 5 minutes of the 20 min restitution period were used as controls and compared with the readouts averaged for the same heart over the period between the 10^{th} and 15^{th} minute after the onset of treatment. This approach allowed to minimize the contribution of heart-to-heart variability into the observed effects of tested compounds on HR and HRV. HR and ECG recorded from the isolated rat hearts perfused with autologous rat blood or washed human red cell suspension were maintained stable over the first two hours of perfusion despite certain variability in absolute values between the individual hearts (Figure S1(http://links.lww.com/JCVP/A238) , Table 1).

Characterization of the HRV included identification of intervals between normal heart beats (N), calculation of maximum, minimum and mean NN intervals (NN_max, NN_min, NN_mean) and the standard deviation of NN intervals (SDNN). The latter reflected overall variation. The square root of the mean of the sum of squares of successive NN differences (RMSSD) reflected short-term variability. Based on these indices hearts were allocated to one of two groups: those with stable rhythm (mean basal SDNN ±2xSD: 5.5 ± 1.8 msec, see Table 1) and the hearts with spontaneous sinus arrhythmia (SDNN>7.3 msec). Graphic representation of the HRV was generated in the form of Poincaré plots (Figure 2). The standard deviation of the NN intervals variability perpendicular (SD1) or along (SD2) the line of identity (SD1) was calculated to characterize the short-term and the long-term variability respectively (30) The ECG time intervals measured included RR interval, PR interval, QRS duration, and QT interval. The QT interval was corrected for differences in heart rate using Bazett formula ($QTc = QT/\sqrt{RR}$).

2.4 Evans Blue as a marker of capillary perfusion

Stock solution of Evans Blue (6% in PBS) was prepared and added to blood used for coronary perfusion reaching final concentration of 0.006% 60 min after the onset of coronary perfusion in the presence or absence of either 50 μM memantine or 300 μM homocysteic acid. Blood with Evans blue was allowed to circulate for 5 min and then perfusion was stopped and the hearts were snap-frozen in isopentane and stored at -80°C. Cryosections (7 μm) of the ventricular tissue were prepared and rapidly dehydrated as described elsewhere (31). Efficacy of capillary perfusion was then assessed as the number of Evans Blue-positive capillaries using fluorescent microscopy (Axiovert 200 M, Carl Zeiss, excitation and emission wavelength of 546 and 590 nm respectively). Quantification of the number and size of perfused capillaries was performed using MCID Analysis image analysis software, MCID.

2.5 Tissue ion content

After 1h of the perfusion hearts were cooled down and perfused with ice-cold solution containing 320 mM sucrose and 20 mM imidazole (pH7.4) for 5 min with the speed of 3 ml/min. Then apex of the heart was dried at 80 ^{o}C chamber for at least 3 days until water was completely evaporated from the sample. Then samples were wet burned in 70% ultrapure HNO_3 and Na^+ and K^+ sample content was determined using flame photometry (IL-943, Instrumentation laboratory, Bedford, MA and M410 Flame Photometer, Sherwood Scientific Ltd, UK) and normalized to the dry weight of the tissue sample.

2.6 ATP and GSH tissue content

Myocardium tissue parts containing both ventricles and a septum were snap-frozen in liquid nitrogen immediately after dissection for the storage. Frozen samples were then homogenized and deproteinized in 5% TCA. Then homogenate was centrifuged (16 000g, 10 min), supernatant was collected into the separate tube and pH was adjusted to 7.5 with saturated TRIS solution. ATP concentration in the solution was measured using an ATP Bioluminescent Assay Kit (Sigma-Alfrich) and resulting luminescence intensity was measured using a Sirius luminometer (Bredford Detection Systems, Germany). ATP concentration was then normalized to the wet weight of tissue sample. GSH was measured in the same deproteinized homogenate using Ellmann's reagent and glutathione reductase (see (32)), GSH concentration was then normalized to the wet weight of the tissue.

2.7 Isolation of the sarcolemmal membranes from atria and ventricles and their utilization for ^{3}H-MK-801 displacement experiments

Hearts were harvested from the male and female Wistar rats (250-350 g) and ventricles and atria used for the isolation of the sarcolemmal membranes as described elsewhere (33). Briefly, frozen atrial and ventricular tissue was homogenized in liquid N_2 and then mixed with a buffer containing 600 mM sucrose, 10 mM imidazol-HCl buffer adjusted to the pH 7.0 in a ratio 3.5 ml/g tissue using standard glass homogenizers. The obtained homogenates were centrifuged at 12 000*x*g for 30 min at +4 ^{0}C. The resulting pellets were discovered and supernatants were diluted with a KCl-MOPS buffer (140 mM KCl and 10 mM MOPS-KOH buffered to the pH of 7.4) to the ratio of 5 ml per 1 g of tissue. The suspension was centrifuged at 95 000*x*g for 60 min using Sorvall Discovery 90 ultracentrifuge and the obtained pellet enriched with sarcolemmal membranes re-suspended in KCl-MOPS buffer and layered over the 30% sucrose solution containing 0.3 M KCl, 50 mM $Na_4P_2O_7$ and 0.1M Tris-HCl (pH 8.3). The membranes were collected, diluted with 3 volumes of KCl-MOPS buffer and sedimented by centrifugation at 95 000*x*g for 30 min and suspended in KCl-MOPS buffer. All procedures were performed on ice and centrifugation performed at 4^{0}C. So obtained sarcolemmal membranes were re-suspended in assay buffer containing (in mM): 118 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 5 $NaHCO_3$, 20 HEPES, 1.2 KH_2PO_4, 2.5 $CaCl_2$, 11 glucose, pH 7.4 and used immediately after the isolation. Membranes were pre-incubated with 3 nM [^{3}H]-MK-801 for 20 min and thereafter exposed to PCPBs (MK-801, ketamine, memantine), eliprodil, or Zn^{2+} at various concentrations for 60 min. Thereafter, membranes were sedimented on a nitrocellulose filter and washed 2 times with 5 mL of assay buffer and the filters with membrane pellets on them were dissolved in 10 ml of Hionic-Fluor (Packard) scintillation fluid. The amount of [^{3}H]-MK-801 bound to the membranes was assessed using TRI-CARB liquid scintillation analyser (Packard, Canada).

[^{3}H]-MK-801 binding rate to the sarcolemmal membranes (SM) pre-incubated with 2 nM or 50 µM of $ZnCl_2$ for 60 min was determined. Binding of [^{3}H]-MK-801 at concentrations ranging from 0.3 to 3 nM was added and allowed to interact for 1 min before the sedimentation and washing was performed as described above. The linearity of [^{3}H]-MK-801 binding kinetics over the time period from 10 sec to 20 min was proven in preliminary experiments.

2.8 Autoradiography

NMDA receptor autoradiography was performed as described elsewhere (31). Hearts and brains were harvested, briefly washed in ice-cold PBS, frozen in liquid nitrogen and stored on -80^{o}C before use. After preparation, 20 µm cryosections of heart and brain were mounted on glass coverslips and dried at RT. The sections were then rinsed in sucrose buffer (190 mM sucrose, 50 mM Tris-HCl, pH 7.4) and allowed to air-dry. Thereafter sucrose buffer solution containing 300 µM NMDA and 300 µM glycine, 30 nM [^{3}H]-MK-801 (20.5 Ci/mmol) was applied on sections. Non-specific binding and [^{3}H]-MK-801 displacement was explored as the buffer was supplemented with 200 µM of either unlabeled MK-801, or memantine, or Ro-25-6981, or 4 mM ketamine, or 200 µM eliprodil, or 200 µM Ro25-6189. Following 20 min of incubation at RT (25 ^{o}C) the sections were washed twice with 250 ml sucrose buffer at RT, dried and placed on X-ray Kodak film for 4 weeks. Developed films were scanned and converted into digital images which were then analyzed using MCID Image analysis software (InterFocus Imaging Ltd, Cambridge, UK).

Radiolabeled antagonist binding assay was also used ex vivo as 30 nM [^{3}H]MK-801 was administered intracoronary and allowed to circulate for 100 min. Accumulation of the [^{3}H]MK-801 in the heart and its extraction from blood was monitored. The hearts were washed free from blood using ice-cold isotonic sucrose buffer and immediately frozen in liquid nitrogen thereafter. Series of 10 µm cross-sections through the whole organ were prepared from aorta to apex and dried out at RT on glass slides. X-ray Kodak film was exposed to those slides during 2 months, and then film was developed and digitized by the same way as described above.

2.9 Adult rat cardiomyocytes isolation

Cardiomyocytes were isolated as previously described (34), in short: Adult rat hearts (N=7) were harvested and perfused via aorta with a solution containing (mM) NaCl 137, KCl 5, HEPES 20, $MgCl_2$ 1.2, glucose 15, and NaH_2PO_4 1, with pH 7.4 at 37°C (solution A). After 2 minutes of perfusion, the perfusion solution was switched to a solution containing (mM): NaCl 130, KCl 4.8,

$NaHCO_3$ 25, $MgCl_2$ 1.2, glucose 12.5, and NaH_2PO_4 1.2 (solution B) with the type 4 Collagenase (Worthington, ~100 U/ml) and 1 mg/ml BSA for 30 minutes. Solution B without collagenase, but with 100 μM $CaCl_2$ and 1 mg/ml BSA was then used to wash the hearts free from collagenase for several minutes. The hearts were then minced and gently agitated to separate the cells. Debris was removed by filtration through a 200 μm mesh filter.

2.10 Photometric recording of action potential duration in isolated adult rat cardiomyocytes

Photometric measurements were performed as described elsewhere (35). Ventricular cardiomyocytes freshly harvested from adult Wistar male rats were loaded with potential-sensitive dye di-8-ANEPPS (5μM, 10 min). After washing free from the extracellular dye with Tyrode the cells were mounted on an inverted microscope (TE2000-U, Nikon, Tokyo, Japan) and paced with the frequency 1 Hz with a field stimulator (MyoPacer, IonOptix). Photometry data were analyzed using Igor Pro software to obtain the values foraction potential duration at 30, 50 and 80% repolarization (APD30, APD50 and APD80). Experiments were repeated using cells from seven independent cardiomyocyte preparations.

2.11 mRNA isolation and qPCR analysis

RNA was isolated from the rat brain, left and right ventricles, septum, left and right atria and a region containing mitral, tricuspid, aortic and pulmonary valves and isolated ventricular cardiomyocytes as described elsewhere (36). cDNA was synthesized from RNA samples by "High Capacity RNA-to-cDNA Master Mix", Applied Biosystems. "TaqMan® Gene Expression Master Mix". Sixty cycles at 95°C for 15 s and 60°C for 1 min were performed using 7500 Fast Real-Time PCR System, Applied Biosystems. Rat GAPDH was used as a reference. Triplicate readings were taken and the average was calculated. The results were normalized to a randomly selected value obtained for the control sample, which was accepted as equal to 1.

2.12 Protein detection by Western Blotting

Rats were anesthetized and euthanized as described above. Hearts were rinsed in ice-cold PBS, cut into pieces and rinsed again. Tissue was immediately homogenized on ice in sucrose buffer containing 0.6 M sucrose, 10 mM imidazole, 1/100 inhibitors cocktail III, pH 7.0 in a ratio of 1 g of tissue in 3.5 ml of buffer. Homogenate was centrifuged (10000 x g, 5 min, 4 ^{o}C) and supernatant then was mixed with Laemli sample buffer. Proteins were separated on a 7.5% acrylamide gel and

transferred to nitrocellulose membrane. TBST (0.1% Tween) with 5% dry milk was used for membrane blocking. Staining was performed with the following primary antibodies: mouse monolconal anti-GluN1 antibody, and rabbit polyclonal anti-GluN2C antibodies (NB300-118 and NB300-107, Novus Biologicals), and rabbit polyclonal anti-GluN2A and mouse monoclonal anti-GluN2B antibodies (ab14596 and ab28373, Abcam). After triple washing of the membranes in TBST the membranes were incubated for 1 h at room temperature with suitable horseradish peroxidase-conjugated secondary antibodies (1:1000 dilution; anti-mouse, Jackson ImmunoResearch Laboratories, West Grove, PA). The enhanced chemiluminescent detection Western blotting system (Fujifilm LAS-3000 System, FUJIFILM Life Science) was used for detection of protein.

2.13 Statistical analysis

Data are presented as means ±SEM if not stated otherwise. Data analysis and statistics were performed using GraphPad Instat Software. Normality test was performed followed by either one-way two-tailed ANOVA tests followed by the Bonferroni post-test, or either Mann-Whitney or Wilcoxon tests depending on the distribution type. The level of significance was accepted at $p<0.05$. Correlation analysis performed for analysis of left ventricular pressure and heart rate variability was performed using linear (Pearson) correlation test.

3 Results

3.1 Impact of cardiac NMDARs into the regulation of autologous rat heart rate (HR) and heart rate variability (HRV)

The effects of intracoronary supplementation of NMDAR agonists (500 µM of NMDA, Glu or HCA) on HR and HRV were tested using autologous rat blood-perfused heart model. Twenty five hearts out of forty tested recovered normal basal rhythmicity prior to the stimulation with the agonists whereas the fifteen (37.5%) exhibited basal increased HRV. In both groups stimulation of the NMDARs did not significantly affect mean inter-beat interval (NNmean) (Table 1). Thirteen hearts responded to application of agonists with gradual increase of the heart rate, Further nine hearts developed dysrhythmic bradycardia (Figure S1,(http://links.lww.com/JCVP/A238) A-D). As blood plasma contains unknown quantities of a variety of potential NMDA receptor agonists (Glu,

Asp, Gly etc.), basal activity of the NMDARs could not be controlled in blood-perfused heart settings.

In order to estimate the half-activation doses for the specific (NMDA and D-Serine) as well as physiological (glutamate and glycine) agonists we have switched to perfusing rat heart with suspension of human RBC (required for sufficient O_2 supply) in plasma-like medium with fixed concentrations of the agonists of choice (Figure 1A). Doses of NMDA and D-Serine required to induce half-maximal tachycardic response (shortening of the RR interval) were 1.55 and 2.1 mM respectively. Red cells are the source of Glu and Gly releasing these amino acids as they lyse in contact with peristaltic pump. Thus dose-dependence of HR (RR intervals) and HRV (RMSSD) responses to Glu and Gly was studied using hearts perfused with red cell-free KH medium supplemented with fixed doses of equimolar Gly:Glu mixture (Figure 1B). EC_{50} for Glu and Gly was 10-fold lower than that of NMDA and D-Ser (149 μM of equimolar mixture) (Figure 1B). Maximal increase in HR produced by the stimulation of NMDARs did not exceed 30% of basal level.

HRV was enhanced in the presence of NMDA receptor agonists both in blood and buffer-perfused models (Table 1, Figure 1B). Regular as well as irregular arrhythmic patterns of inter-beat intervals were produced (Figure 2 A-C and Figure 2S). For glutamate and glycine mixture, EC_{50} in KH buffer perfusions was 271 μM.

3.2 HR and HRV in rat heart exposed to NMDAR antagonists

The effects of blockers of the NMDARs on the HR and HRV were further explored. A selection of antagonists binding within the channel pore (PCP binding site: MK-801 (50 μM), memantine (50 μM), and ketamine (420 μM)) and to the modulatory polyamine binding site (60 μM eliprodil, 50 μM Ro25-6981) was applied intracoronary in autologous blood-perfused rat heart model. Irrespective of the class, all antagonists produced a pronounced decrease of the HR in both rhythmic and arrhythmic hearts (Table 1, Figure 2C-G, Figures S1(http://links.lww.com/JCVP/A238) G and S2(http://links.lww.com/JCVP/A239)A). Zinc ions are known to bind to the amino terminal domain of the GluN2A and 2B subunits producing voltage-dependent block in the receptors formed by GluN1/GluN2 subunits, but potentiating the glycine-binding GluN1/GluN3 type of NMDARs (37-40). IC_{50} for $ZnCl_2$ ranges from 10 nM to 30 μM depending on the subunit composition of the

receptor. When used at 10-50 μM concentrations, $ZnCl_2$ induced tachyarrhythmia that could not the suppressed by the pore-targeting antagonist MK-801 (Table 1, Figures S1H and S2A).

Supplementation of competitive antagonists targeting glycine and glutamate binding sites within amino terminal domain (L-701 and D-APV) at 500 μM concentration to blood perusing cardiac coronaries had no effect on the HR or HRV.

Along with bradycardia, NMDAR antagonists were effectively suppressing pre-existing sinus arrhythmia. Anti-arrhythmic effect could be observed for pore-targeting and polyamine site-directed antagonists (Table 1, Figure 2 Figures S1G and S2A) whereas Zn^{2+} exhibited a prominent pro-arrhythmic activity (Table 1, Figures S1H and S2A). Along with increase in HRV caused by short-term ischemia-reperfusion during heart harvesting, antagonists were effectively suppressing arrhythmias induced by hyperactivation of NMDARs (Figure 2C, Figure S2,(http://links.lww.com/JCVP/A239) B). Arrhythmias triggered by Zn^{2+} supplementation were making the hearts insensitive to the action of pore-targeting antagonist MK-801 (Figure S1,(http://links.lww.com/JCVP/A238) H).

Anti-arrhythmic activity of antagonists observed in hearts of young (1-3 months old) rats was confirmed in four hearts of senescent (1-2.5 years old) animals presented with ventricular fibrillation shortly after the beginning of perfusion with autologous blood. Intracoronary administration of ketamine (1 mM, n=2) or memantine (50 μM, n=3) restored rhythmic autologous contractile function in fibrillating senescent hearts, as exemplified in Figure 3, although at substantially lower HR (50-120 bpm vs. 150-200 bpm in blood-perfused senescent hearts).

As shown in Table 2 none of the antagonists is 100% selective interacting with NMDAR alone. We have thus compared the action of NMDARs antagonists with the effects of L-type calcium channel blockers (CCBs) diltiazem and verapamil on HR and HRV. In contrast to the NMDAR antagonists, intracoronary administration of CCBs induced severe bradycardia (Table 1) and suppressed sinus node activity switching the hearts to ventricular escape rhythm (Figure 2H,I Figures S1I and S2A, Table 1).

3.2 ECG

RR intervals recorded of blood-perfused hearts remained unchanged after the stimulation of NMDAR agonists (Table 1, Figure 4A, F). ST amplitude decreased in all measurements both in

blood- and KH-perfused hearts (Figures 4A, 5A-B). This response is characteristic for ischemic heart. We have thus tested the efficiency of capillary perfusion in hearts perfused with blood supplemented with saturating concentrations of agonists HCA or NMDA using Evans Blue (Figure 5C,D). As follows from the representative image in Figure 5C capillary perfusion was severely compromised in ventricular tissue of the hearts stimulated with agonists as the number of perfused capillaries dropped by two thirds (Figure 5D). Administration of antagonist improved it. Inhibition of the cardiac NMDARs was associates with increase in the mean size of capillary lumen compared to the control heart (Figure 5D).

Bradycardia caused by the channel pore-targeting antagonists was associated with prolongation of QTc interval (Figure 4B,I). A tendency of PR and QRS complex prolongation did not reach statistical significance (Figure 4G,H). Antagonists binding to the polyamine site antagonists significantly prolonged QRS complex, but QTc interval remained unchanged (Figure 4H,I). $ZnCl_2$ and L-type calcium channel blockers didn't affected ECG wave intervals but caused premature ventricular contractions (Figure 4D,E).

3.3 Action potential duration in isolated ventricular cardiomyocytes

To evaluate the contribution of cardiomyocytes into the responses of rat heart to the stimulation or inhibition of the NMDARs, ventricular cardiomyocytes were isolated for the functional test. Cells while paced at 1 Hz frequency developed action potentials which were detected by a ratiometric read-out of the fluorescent dye Di-8-ANEPPS. Cardiomyocytes treated with similar concentration of MK-801 as was used for the whole heart perfusion developed a significant prolongation of the action potential (Figure 6).

3.4 Expression and localization of different NMDA receptor subunits

The presence of the NMDAR subunits at the protein level in whole heart homogenate was explored using immunoblotting. Total rat brain protein extracts were used as a positive control. Cardiac GluN1, GluN2A, 2B and 2C subunits recognized by the corresponding antibodies appeared to be similar to those in the brain (Figure 7A). The signal for GluN2D subunit was identical for in rat brain and heart-derived protein, but its weight (170 kDa) was somewhat higher than 135 kDa

reported for this subunit (data not shown). Expression of GluN2B protein increase in hearts of rats as they aged (Figure 7B).

Expression pattern of GRIN genes encoding the NMDAR subunits was mapped in rat heart chambers, septum, and in the valvular region using RT-PCR and compared to that in the brain. As shown in the Table 3, GluN1 and GluN2A expression was above detection threshold in atria only, whereas transcripts of GluN2B and 2D, and that of GluN3A genes were detected in all regions of the heart. GluN2C was only detected in septum and in the left atrium. In adult rat brain, prominent expression of the GluN1, 2A and 2B was found. In rat heart most abundant were the transcripts coding for the glutamate-binding GluN2D and glycine-binding GluN3A subunits of the receptor. In cardiomyocytes isolated from ventricular tissue and septum, GluN2B, GluN2D, and GluN3A transcripts were detected.

3.5 Cardiac NMDA receptor pharmacology

Distribution of NMDARs within the heart perfused with blood was tested (Figure 8A,B). Autologous rat blood filling perfusion circuit was supplemented with 30nM [^{3}H]MK-801 (Ki 2-14 nM, Kd 5-26 nM, see supplementary Table 1, http://links.lww.com/JCVP/A240) and allowed to perfuse the coronaries for 100 minutes. Aliquots of blood were taken during perfusion to assess the antagonist binding kinetics. Hearts accumulated 70% of [^{3}H]MK-801 during the first 20 min of perfusion (Figure 8B). The hearts were then washed free from non-bound [^{3}H]MK-801 and frozen. Cryosections were then prepared and placed on the film for autoradiographic detection of [^{3}H]MK-801 binding localization. Experiments were performed with hearts of young (1-3 months-old, N=3) and senescent (over a year-old, N=3) rats. Density of active NMDAR within the heart was chamber- and age-dependent being particularly high in the atria of young animals and in ventricles of senescent rats (Figure 8A).

Affinity of cardiac NMDAR to selected antagonists was assessed using [^{3}H]MK-801 displacement assay and compared to that in rat brain. To do so, brain and heart tissue cryosections were pre-incubated with agonists-containing medium (300 μM NMDA and 300 μM glycine) supplemented with NMDAR antagonists and then the number of active receptors was measured by [^{3}H]MK-801 binding assay. The cardiac NMDARs were effectively inhibited by pore-targeting MK-801 and memantine, as well as by polyamine site-binding eliprodil and Ro25-6981 (all in 200 μM final concentration) as well as somewhat less effectively by 4 mM ketamine (Figure 8C). Inhibition of the cardiac receptors by memantine was even more pronounced compared to that in the brain. Eliprodil

targeting GluN2B subunit of the receptor was not able to block the brain derived NMDARs as expression of this subunit is very low in most the regions of adult rat brain (41).

Titration curves were produced to determine dissociation constants for memantine, ketamine and MK-801 for sarcolemmal membranes using [^{3}H]MK-801 displacement technique. Two types of binding sites were clearly detected for MK-801 and ketamine, whereas the curve for memantine could be fitted with a single sigmoidal function. Cardiac NMDARs showed very high sensitivity to memantine with a Kd of 1.3±0.4 nM followed by MK-801 with a Kd of 1.8±0.3 nM and ketamine with a Kd of 1200±400 nM (Figure 8D). Sensitivity to the blockers did not differ between atria and ventricles. Molecular identity of the second low affinity binding site for MK-801 and ketamine remains to be explored. Supplementation of eliprodil at 50 μM concentration interfered with displacement of [^{3}H]MK-801 by non-labeled MK-801 decreasing Kd from 1.8±0.3 to 6.2±1.7 nM. In line with previous observations (Figures 4D, S1H and S2A, and Table 1) $ZnCl_2$ in nanomolar range activated the receptor promoting [^{3}H]MK-801 binding to the sarcolemmal membranes. At high doses it suppressed the NMDARs and interfered with [^{3}H]MK-801 binding within the open channel pore (Figure 8E).

3.6 Higher sensitivity of senescent hearts to agonists of NMDARs and cardioprotective potential of antagonists in hypoxic senescent hearts

Earlier on we have shown that hearts of young rats (3-6 month) are capable of sustaining contractile function when hemoglobin oxygen saturation of blood used for coronary perfusion is reduced from 98 to 35% for at least 40 min (Table 4, (22, 42)). Arrhythmia was developing only in 25% of cases within 40 min of severely hypoxic conditions, no stunning was observed in any of the hearts. Senescent hearts on the contrary were sensitive to shortage in oxygenation and developed arrhythmias within 15 min of non-ischemic global hypoxia (Table 4). Stunning was induced by hypoxia in 20% of hearts in 15 min and in 60% of them within 40 min of perfusion with poorly oxygenated blood (Figure 9). These events were observed as cardiac NMDARs were activated by endogenous plasma-borne agonists. If HCA or NMDA were supplemented to achieve maximal stimulation of the receptors, hearts extracted from young animals developed arrhythmias in 36 % of cases and stunning in 18% of cases after 40 min of perfusion with deoxygenated blood (Table 4). These observations are in line with the data on the characteristic ST sagging and compromised capillary perfusion of the myocardium induced by the cardiac NMDAR hyperactivation (Figure 5) aggravating hypoxic condition even further.

Following up on this observation we have speculated that blocking of the receptor associate with improved capillary perfusion (Figure 5C,D) could support senescent heart failing to sustain contractility under hypoxic conditions. Indeed, poor tolerability of hypoxia could be substantially improved if the channel pore-targeting antagonists MK-801 or memantine were added intracoronary and none of senescent hearts underwent stunning over 40 min of hypoxic exposure (Figure 9). As mentioned above (Table 1 and Figures 1, 2, 4, S1 and S2), administration of antagonists induced bradycardia. The incidence of arrhythmia decreased from 80% to 21% after 15 min of hypoxic perfusion and from 100 % to 47% within 40 min of reduced O_2 supply (Table 4).

We further explored the mechanisms of cardioprotective action of MK-801 and memantine. Earlier on we have reported an increase in tissue Na^+ content in the left ventricle and a decrease in the Na,K-ATPase function in young rat hearts exposed to non-ischemic hypoxia (22, 42, 43). In line with that both young and senescent hearts responded to hypoxia with an increase in tissue Na^+ content that did not reach statistical significance due to the high degree of heart-to-heart variation (Figure 10). Intracoronary administration of the NMDA receptor antagonists (memantine and MK-801) significantly reduced tissue Na^+ in both normoxic and hypoxic hearts independent on the animal age. Swelling in hypoxic senescent hearts was more pronounced as Na^+ uptake was not followed by the loss of K^+. Pore-targeting antagonists triggered the extrusion of K^+ along with the suppression in Na^+ uptake improving capillary perfusion in senescent myocardium (Figures 10 and 5C-D).

Modulation of the cardiac NMDAR activity had an impact on the myocardial ATP content (Figure 11). Development of arrhythmia was associated with ATP depletion in young hearts. Early decrease in contractile function and stunning of hypoxic senescent hearts occurred before tissue ATP content was impacted (Figure 11) suggesting that ATP depletion was not the cause of Na^+ accumulation and stunning. In senescent hearts that maintained contractile function over 40 min of hypoxia reduction of ATP levels was observed. Intracoronary administration of memantine prevented ATP depletion in these hearts (Figure 11). As a result of reduction in tissue perfusion ATP content decreased in young hearts subjected to hypoxia in the presence of HCA, but not in the presence of MK-801 (Figure 11).

Tachyarrhythmia in the hearts of young animals triggered by the co-administration of agonists and deoxygenation was associated with GSH depletion that could be prevented with antagonists of the NMDARs. Bradycardia in hypoxic senescent hearts produced an increase in total GSH content that was insensitive to the NMDAR antagonists (Figure 11).

4. Discussion

4.1 Characterization of cardiac NMDARs

Whereas the ability of NMDAR agonists and antagonists to alter the heart rhythmicity and modulate severity of ischemia-reperfusion injury has been acknowledged recently (18, 44), the subunit composition, pharmacological properties and molecular mechanisms of cardioprotection by the NMDAR antagonists remained poorly understood. Mitochondrial dysfunction was considered as one of the mechanisms of myocardial damage following NMDAR hyperactivation (45-49). Ca^{2+} overload in cardiomyocytes was suggested to be involved in pathological response to the stimulation with high doses agonists of the NMDAR (47).

We have shown that subunit composition of cardiac NMDARs differs substantially from that in the brain (50) and resembles that in erythroid precursors and red cells (51). Mapping of the NMDAR subunits expression pattern within the heart chambers revealed that cardiac receptors formed by the GluN3A-GluN2D/2B with lower abundance of GluN1, 2A and 2C that are present at low levels in certain areas of the heart only (Table 3). Expression levels of distinct subunits (GluN2B) and the NMDAR abundance in atria and ventricles appeared to be strikingly age-dependent (Figures 7B, 8A). Age-dependence of GluN2B expression was reported earlier on (52). Furthermore, GluN2B subunit of the NMDAR was reported to form complex with ryanodine receptor RyR2 in the heart (19). This subunit was claimed to respond to mechanical stimulation (53) making the function of cardiac NMDAR potentially mechano-sensitive and linking it to the contractile function. In-depth investigation of the mechano-sensitivity of cardiac NMDARs was out of the scope of this study, but seems to be of clinical relevance.

Pharmacological profile of the cardiac NMDARs is in agreement with the data on NMDAR subunit composition. Biphasic dose-response curves obtained for PCPBs interaction with sarcolemmal membranes (Figure 8D) were similar to those reported for the neuronal NMDARs earlier on (see references to the supplementary Table 1, http://links.lww.com/JCVP/A240). GluN2B-specific PAAs eliprodil and Ro25-6981 were equally efficient in displacement of [^{3}H]-MK-801 (Figure 8C) and producing bradycardia (Figures 2G, 4F, S1F, and S2A, Table 1) as were PCPBs (Figures 8C,D, 2D-F, 4F S1E, and S2A-B). The absolute IC_{50} values obtained for neuronal and cardiac NMDARs using [^{3}H]-MK-801 displacement in membranes are not always within the same range (Table 2, suppl Table 1, http://links.lww.com/JCVP/A240). Affinity of the cardiac NMDARs to memantine is

three orders of magnitude higher than that of neuronal receptors (Table 2, suppl. Table 1, http://links.lww.com/JCVP/A240). This suggests that cardiac NMDARs, not the neuronal receptors are the primary target of memantine in rats. Whether this hold true for patients with Alzheimer's disease remains to be explored. Furthermore, rat cardiac NMDARs respond to ketamine which is commonly used in veterinary practice.

Differences in receptor-antagonists interactions reflect variance in subunit composition of the cardiac and neuronal NMDARs. Whereas adult rat neuronal NMDARs are composed of GluN1, 2A and 2C, cardiac NMDARs are built of GluN3A, GluN2D, GluN2B is less abundant, and GluN1, 2A, and 2C are present at low levels in certain areas of the heart only (Table 3). These molecular settings explain the dual (pro-agonistic at low doses and antagonistic at high concentrations) action of Zn^{2+} on the cardiac NMDARs (Figure 8E). Earlier the stimulatory effect of Zn^{2+} on the receptors composed of GluN3A-GluN1 and its inhibition by interaction with the GluN2B subunits was reported (39, 40).

4.2 Physiological and pathophysiological roles of cardiac NMDARs

Our data indicates that a substantial number of the cardiac NMDARs are active in blood-perfused rat heart. Their activity is maintained by endogenous agonists of which glutamate (35-200 μM) and glycine (180-310 μM) (55-58) are most abundant in plasma and the most potent activators of the cardiac NMDARs (Figure 1B). Glutamate EC50 concentration reported for the neuronal NMDARs is 100 μM (59) whereas for the HR response the EC50 was 149 μM (Figure 1B). Recordings of ionic currents mediated by the NMDARs are required to assess EC50 of the cardiac NMDARs more precisely. Paracrine release of glutamate from the cardiac muscle, where it is highly abundant (30 $\mu mol/mg_{protein}$), hemolysis, and other processes causing alterations of plasma glutamate levels may thus contribute to the development of tachycardia and arrhythmia. Glutamate is currently extensively used as an essential component of cardioplegic solution, where its concentrations may exceed 20 mM. Exogenous glutamate is effectively extracted by cardiomyocytes, and taken into the mitochondria where it is fed into the tricarboxylic acid cycle via malate-aspartate shuttle supporting respiration, particularly in ischemic heart (60). In rats, these supra-physiological glutamate concentrations were shown to acutely reduce infarct size during the first minutes of reperfusion, but did not improve hemodynamic recovery (33, 54). Our observations suggest that exogenous glutamate has more than one target, acting on the pacemaker function (HR, Table 1), conductive

system (QRS interval, Figure 4H) and ventricular repolarization (QT interval, Figure 4I) along with facilitation of mitochondrial respiration.

Based on our data we cannot precisely identify the cause of arrhythmia triggered by hyperactivation of the cardiac NMDARs. However, Na^+ accumulation and oedema promoted by the administration of saturating doses of the NMDAR agonists (Figures 5 and 10) may be one of the causes. ATP depletion occurring in young hearts upon combined treatment with the NMDAR agonists and hypoxia as well as in hypoxic senescent heart contributed to the development of arrhythmia and stunning (Figure 11). In the brain of rats in which severe hyperhomocysteinemia was induced by administration of homocysteine, decreased activity of the Na,K-ATPase was observed along with mitochondrial dysfunction (61-63). Investigation of the action of high doses of homocysteine on the mitochondrial function in cardiomyocytes was reported earlier (64). The role of Na,K-ATPase in myocardial dysfunction caused by the NMDAR agonists needs further investigation. The ability of NDMAR antagonists to suppress arrhythmias triggered by ischemia-reperfusion and by the NMDAR hyperactivation points towards their therapeutic potential.

At present it is impossible to discriminate between the action of antagonists on the action potential and the excitation propagation (Table 1 and Figures 4 and 6) and their ability to improve capillary perfusion (Figure 5). Administration of antagonists (both PCPBs and PAAs) has been shown to reduce brain oedema associated with deoxygenation (65, 66). The obtained data reveal importance of cardiac NMDARs activation state in autonomous control of HR and HRV, function of conductive system (QRS intervals), and ventricular repolarization (QT). Anti-arrhythmic properties of the pore-targeting PCPBs were associated with a prolongation of QTc intervals (Figure 4B,I). These findings were supported by the data on prolongation of APD30 and APD50 in isolated adult rat cardiomyocytes in the presence of MK-801 (Figure 6). Polyamine site-directed PAAs targeting the GluN2B subunit didn't affect QTc intervals, but prolonged duration of QRS complex (Figure 4C,H,I). Expression of this subunit appeared to be amplified with aging, suggesting that antiarrhythmic effects of PAAs will be more pronounced in senescent rat hearts (Figure 7B). Reduction in abundance of this subunit in adult and senescent brain (Figure 8C, and (67, 68)) makes eliprodil action in adult rats largely restricted to the cardiac NMDARs. In contrast to the CCBs, NMDAR antagonists are anti-arrhythmic whereas verapamil and diltiazem were pro-arrhythmic, producing a HRV pattern resembling 2nd degree AV block or sinus exit block (Figures 4E and S2A).

Conclusions

Taken together, these findings indicate that the NMDAR agonists and antagonists are potent modulators of autonomous rat heart function. The observed responses are clearly distinct from those produced by the CCBs and are mediated by a set of cardiac NMDARs. The obtained results turn cardiac NMDARs into potentially interesting targets for prevention and treatment of malignant arrhythmias and heart ischemia.

Funding

This study was supported by the SNF grants 1112 449 and 310030_124970/1 to AB and by Saarland University's internal funding program HOMFOR (to QT and LK).

Acknowledgements

We thank Alexander Kosenkov for his help with analysis of autoradiogaphic images.

Conflict of interests: none declared

Figure legends

Figure 1. The effects of agonists on heart rate (HR) and heart rate variability (HRV) in autonomously beating rat heart A: Dose-dependence of the effects of specific antagonists NMDA and D-Serine on the beat-to-beat intervals in rat heart perfused with human red cell suspension. Basal HR and HRV were taken as 100%. Numbers are EC50 for each amino acid. B: HR (RR interval) and HRV (RMSSD) in rat hearts perfused with KH solution supplemented with fixed concentrations of equimolar Glutamate-Glycine mixture. Red cells were not used to avoid leakage of these amino acids in the course of cell trauma and lysis caused by the peristaltic pump. Concentrations of amino acids causing half-maximal effect are shown. Data shown are means of 4 independent experiments ±SD.

Figure 2. Representative Poincare plots for the young rats' hearts perused with autologous blood supplemented with the NMDAR agonists and/or antagonists as well as with L-type Ca^{2+} channel blockers. X-axis represents the NN Intervals (RR interval duration (msec) between normal sinus beats at fixed time point) and Y-axis shows the next NN interval duration. A,B, and C: Pro-arrhythmogenic effects of stimulation of the cardiac NMDARs with 300 μM NMDA, HCA or Glutamate respectively. Arrhythmia induced by stimulation of the heart with Glutamate was suppressed by 50 μM MK-801 (C). Panels D, E and F exemplify anti-arrhythmic action of pore-

targeting antagonists 50 µM MK-801, 50 µM memantine or 1 mM ketamine respectively. Arrhythmias originated from a brief period of ischemia-reperfusion during the heart harvesting. G: Anti-arrhythmic action of eliprodil targeting the PAA site of the receptor. H and I: pro-arrhythmogenic action of the blockers of the L-type Ca^{2+} channels diltiazem and verapamil (10 and 5 µM respectively). Quantification of the data and statistical analyses are shown in Table 2.

Figure 3. An example of the rescue of contractile function in the fibrillating autologous blood-perfused heart by administering of memantine (50 µM, n=3) and ketamine (1 mM, n=2). Shown in the figure are the heart rate after contractile function was restored.

Figure 4. Effects of agonists and antagonists of the NMDAR and L-type Ca^{2+} channel blockers on the ECG intervals. A: Waterfall diagrams of the changes in ECG in agonist-treated heart (300 µM of equimolar Gly-Glu mixture). B: Responses to the PCPBs (MK-801 (50 µM), memantine (50 µM), ketamine (1 mM)). C: Responses to the PAAss (eliprodil or Ro25-6981(60 µM each)). D: Responses to $ZnCl_2$ (50 µM). E: Responses to the CCBs (Diltiazem (10 µM), Verapamil (5 µM)). Statistics on the RR (F), PR (G), QRS (H), and QTc (I) intervals for all the compounds used. Presented are box plots (medians, of 6-25 experiments and variance) of the values of the respective intervals overlaid with dots representing the values recorded for individual hearts. Paired t-test was used for statistical analysis. "ns" stands for not significantly different, and *, ** and *** denote $p<0.05$, 0.01 and $p<0.001$, respectively.

Figure 5. Ischemia induced by the hyperactivation of cardiac NMDARs. A: Original recording so the ECG wave before (upper panel) and after (lower panel) the stimulation of the NMDAR with the agonist (NMDA) red arrow pointing to the ST interval and B: ST sagging quantification, the number above is a p-value for two tailed paired t-test. C: Representative images of capillary perfusion efficacy in control heart, after the NMDAR stimulation with 300 µM HCA and the one perfused with blood supplemented with 50 µM memantine. Perfused capillaries are visualized by Evans Blue penetration into the microvasculature. D: Quantification of the number of perfused capillaries and the mean size of capillary lumen in control hearts and the ones with activated or inhibited NMDARs. Data are means of 15-58 images (numbers inside the bars) of cryosections obtained from ventricles and apex regions of 3-4 hearts ±SD. Two-tailed unpaired t-test (following the normality test) was used for statistical analysis (p values shown above the bars).

Figure 6. The action of MK-801 on the action potential duration (APD30, APD50 and APD80). Action potential duration after 30, 50 and 80% of repolarization in isolated adult rat cardiomyocytes. 7 animals were used for this experiment, around 100 cells were analyzed for each animal. Dashed rectangles represent cardiomyocytes treated with 40 μM of MK-801, and white rectangles are controls. Averaged APD values (93-97 action potential readouts) obtained for untreated control cells (basal level) were compared with those after MK-801 exposure using by paired t-test. Overlapped original traces of APD from a single cell at baseline and after the MK-801 administration are shown.

Figure 7. Confirmation of the presence of GluN1, GluN2A, GluN2B and GluN2C proteins in rat heart. Rat brain tissue homogenate was used as a control. Panel A shows the representative images in which the subunits were detected by immunoblotting. B: Changes in the GluN2B protein abundance in the heart with age of the animal. Actin was used as a loading control.

Figure 8. Localisation and pharmacological characterisation of the NMDARs. A: Local heterogeneity and subunit composition of cardiac NMDAR. Receptor activity in blood-perfused young (1.5 months old) and old (1.5 years old) rat heart chambers visualized using [3H]-MK-801 localization (autoradiography). B**:** Time-dependence of [^{3}H]MK-801 depletion of plasma of autologous blood used for coronary perfusion. C: Autoradiographic detection of [^{3}H]-MK-801 displacement studies of the antagonists binding to the heart and brain sections. Representative images (left panel) and quantification of displacement (right panel) Data are means ±SD from 4-6 sections. 0 stands for control, NG for NMDA-glycine-treated sections, MK, Mem, Ket Ro25/Eli standing for MK-801, memantine, ketamine, Ro25-6981 and eliprodil. One-way two-tailed ANOVA with Dunnett post-test were used for statistical analysis. False scale is showing the color coding of the signal strength. D: Dose dependent displacement of [^{3}H]-MK-801 by other MK-801, memantine and ketamine in ventricular sarcolemmal membranes (SMs). E: Dual effect of Zn^{2+} on the NMDAR activity in ventricular SMs. Shown are the dose-dependence of the action of Zn^{2+} on the NMDAR activity (detected as [^{3}H]-MK-801binding efficacy) and the rate of [^{3}H]-MK-801 binding rate to the SMs in the presence of 2 nM or 50 μM $ZnCl_2$. Data are means of 3 independent experiments (5 parallel measurements done for each dose) ± SD. *denotes $p<0.05$ compared to the Zn^{2+}-free control.

Figure 9. Original trace of the heart rate recording for the senescent hearts responding to hypoxia with stunning and the rescuing effect of 50 μM MK-801. Statistics for these experiments is presented in Table 4.

Figure 10. Tissue Na^+ and K^+ content in young and senescent hearts exposed to hypoxia in the presence of absence of MK-801 in autologous blood used for coronary perfusion. Values are means of 3-17 experiments (numbers within the bars) ±SD. P-values obtained using two-tailed t-test are shown above the bars.

Figure 11. Tissue ATP and GSH control in young and senescent hearts exposed to hypoxia and/or 300 μM HCA. Senescent hearts are presented as "all hearts" as well as divided into "stunned" and "beating" under hypoxic conditions. Values are means of 3-17 experiments (numbers within the bars) ±SD. P-values obtained using two-tailed t-test are shown above the bars.

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Table 1. Time domain analysis of the heart rate variability for autonomously beating rat hearts exposed to the agonists and antagonists of the NMDARs or to the L-type Ca^{2+} channel blockers

	NN_{max}	NN_{min}	NN_{mean}	SDNN	RMSSD	SD1	SD2	N
Baseline: normal	297.4±16.1	209.7±17.8	266.8±13.0	5.5±0.9	4.58±0.79	2.37±0.44	2.94±0.39	25
Baseline: arrhythmia	696.1±95.1***	188.3±22.6	311.0±18.3*	33.2±4.2***	40.0±6.7***	22.9±5.0***	27.5±5.1***	15
Normal before agonists	300.9±29.7	179.8±32.7	265.6±20.7	5.4±1.4	4.98±1.2	2.1±0.5	3.0±0.6	7
Normal after agonists	386.4±53.2	189.8±37.2	272.8±21.2	20.8±6.8	20.21±7.95	25.7±12.6*	24.6±10.9***	7
Arrhythmia before agonists	633.0±122.0	150.3±20.3	347.3±20.6	35.1±6.1	41.8±12.4	7.53±0.9	13.6±1.4	5
Arrhythmia after agonists	802.0±297.5	234.78±40.42	336.89±15.43	40.44±9.28	51.27±13.66	17.3±1.83	29.27±2.53	5
Normal before antagonists	305.68±22.88	236.79±21.8	277.7±17.6	5.3±1.3	3.9±1.0	2.8±1.1	3.3±0.9	13
Normal after antagonists	370.1±29.86**	329.6±22.2***	346.6±25.1***	4.2±1.2	3.02±0.94	4.24±1.86	5.68±1.84	13
Arrhythmia before antagonists	773.54±138.6	219.09±32.39	296.1±26.6	33.2±6.3	40.77±9.26	33.05±6.49*	31.29±6.42*	9
Arrhythmia after antagonists	423.26±31.86*	246.76±29.74	349.4±32.13*	9.48±1.77**	10.42±2.38*	8.31±3.1**	10.2±2.2**	9
Normal before Zn^{2+}	398.68±37.06	347.15±42	379.57±36.41	6.51±0.91	4.68±1.69	2.81±1.06	5.41±1.02	10
Zn^{2+}	557.4±136.9	241.0±65.3*	343.4±35.9*	28.0±8.0	30.6±18.2	27.6±17.1	22.6±9.9	10
Zn^{2+} and MK 801	478.4±78.8	268.7±34.8	345.8±33.0	19.0±7.4	13.8±5.9	5.8±1.6	1.9±0.6	10
Normal before L-type blockers	349.7±52.6	210.2±9.6	259.3±23.7	4.9±0.9	5.9±1.5	2.2±0.8	3.1±0.4	6
Normal after L-type blockers	543.5±40.2	334.7±90.8	465.4±55.4*	38.4±16.9	51.7±27.0	35.9±16.7	41.8±15.7	6
Arrhythmia, before L-type blockers	1339.0±450.3	206.2±67.2	319.7±20.4	42.8±13.6	61.3±17.8	38.0±19.6	34.0±21.6	4
Arrhythmia, L-type blockers	835.3±338.1	261.1±65.9	410.9±43.1	58.3±36.8	72.01±42.1	36.8±34.5	53.8±50.0	4

Agonists (300 μM NMDA, homocysteic acid, glutamate) and antagonists (MK-801 or memantine (50 μM); ketamine (1 mM); Ro25-6981 or eliprodil (60 μM); 50 μM $ZnCl_2$, and L-type Ca^{2+} channel blockers (diltiazem (10 μM) or verapamil (5 μM)). Shown in the table are the maximal NN interval within 5 min period (NNmax), minimal NN intervals (NNmin), means of NN intervals (NNmean), standard deviation of NN intervals (SDNN), square root of the mean squared difference of adjacent NN (RMSSD; quantitative characteristics of the Poincaré plots (SD1 and SD2). All values are presented as means ±SE. *p<0.05, **p<0.01, ***p<0.005.

Table 2. Inhibitory constants IC50 for the NMDAR antagonists for selected targets

	Ketamine	MK-801	Memantine	Eliprodil
ACh receptors	6-15 ìM	21 ìM	1 - 7 - 11 ìM	-
Opiate receptors	27 ìM	-	-	27 ìM
5-HT receptors	3-30 ìM	-	~1 ìM	-
Ca^{2+} channels	33 ìM	-	-	-
K^+/Na^+ channels	1-20ìM	?	-	-
Neuronal NMDA receptors	10 - 40 ->100 ìM	10-40 nM	1 ìM	1-10 ìM
[^{3}H]MK-801 displacement in neurons	1-2.5 ìM	2-14 nM	0.4-0.7 ìM	
[^{3}H]MK-801 displacement rat in atria	*na*	15.0±4.0 nM, N=15	36.8±9.2 nM, N=15	*na*
[^{3}H]MK-801 displacement rat in ventricles	1.2 ± 0.4 ìM, N=6	1.8 ± 0.3 nM, N=7	1.3 ± 0.4 nM, N=5	6.2 ± 1.7*^{ł} nM, N=7

^{ł} – displacement by MK-801 in a presence of 50 µM eliprodyl. * - $p<0.05$ vs MK-801. N indicates the number of independent experiments. Shown are means ±SEM

Data for the [^{3}H]MK-801 displacement in rat atria and ventricles are generated within this study. Valused for the neuronal NMDARs and the other channels are receptors are obtained from the literature sources listed in the supplementary Table 1

Table 3. Local pattern of NMDAR subunits' expression in rat heart (Δct, GAPDH expression used for normalisation)

Tissue/ cells	*GluN1*	*GluN2A*	*GluN2B*	*GluN2C*	*GRIN2D*	*GluN3A*	*GluN3B*
Brain	**6.18**	**6.42**	6.55	8.42	10.77	8.69	18.62
Septum	-	-	20.9±0.6	21.1±1.0	15.6±1.9	19.1±0.6	-
LV	-	-	20.2±1.9	-	15.2±2.6	17.7±2.6	-
RV	-	-	19.8±2.1	-	15.4±2.9	17.6±3.4	-
LA	20.2±1.4	20.6±0.6	21.1±1.2	20.3±1.8	14.9±1.1	18.2±1.0	-
RA	18.3±1.0	19.1±1.1	19.0±1.0	-	15.3±1.9	18.3±4.1	-
Valves	-	-	20.4±1.8	-	13.6±2.6	11.9±4.2	-
ARCs	-	-	21.5±0.6	-	18.5±0.5	22.0±0.03	-

Presented are values obtained for whole brain homogenate (Brain), various areas of the heart: septum, left and right ventricles (LV and RV), left and right atria (LA and RA), and valvular region (Valves), as well as in fresh-isolated adult ventricular rat cardiomyocytes (ARCs). Presented are means ± SD for 42 hearts and 3 brains. Cells were isolated from 3 different hearts. Hyphen indicates the levels below detection limits (60 cycles).

Table 4. Number of blood-perfused hearts isolated from young and old rats showing severe dysrhythmia and stunning after 15 and 40 min of hypoxia

		arrhythmia		stunning	
	Total N	15 min	40 min	15 min	40 min
Young control	8	0	2	0	0
Young + HCA	11	0	4	0	2
Old control	15	12	15	3	9
Old + ant	19	4	9	0	0

HCA stands for 300 μM homocysteine acid and "ant" for 50 μM MK-801 or memantine